STRUCTURE-ACTIVITY RELATIONS IN TWO NEW SERIES OF ANTIFOLIC ACIDS

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ANTIFOLIC acids† are clinically useful in acute leukaemia (aminopterin, A-methopterin) and in malaria (proguanil, pyrimethamine); but aminopterin and its relatives possess the disadvantages of producing severe side-effects and of evoking drug-resistance[1], which latter can also be a problem with the antifolic antimalarials[2]. It therefore seemed useful to search for new types of antifolic acids, which might allow the development of additional therapeutically useful substances, not cross-resistant with existing types. Taking the 2:4-diaminopteridines known to possess very high antifolic acid activity in various bacterial systems[3–6] as a starting point, two series of compounds—the aryl-azopyrimidines and 8-azapurines—were prepared and investigated. Since both series were readily varied chemically, they allowed structure-activity relations to be explored in some detail. In the account that follows the chemical and biological investigations are described in separate sections and the results then brought together in a discussion of structure-activity relations.

This work was started in the Autumn of 1953, and some of the compounds were described[7] at the International Chemical Congress in 1955, where conversation with Dr. E. J. Modest revealed that somewhat later than ourselves he had prepared and begun to examine some similar compounds. Arrangements were made with Dr. Modest for simultaneous publication and a paper by himself and his colleagues[8] appears alongside our own.

CHEMICAL SECTION‡

INTRODUCTION

Antagonists of folic acid in bacterial systems have been found by various workers amongst the following simple derivatives of 2:4-diaminopteridine; 6:7-diaryl- (I, $R' = R'' =$ aryl)[3,4], 7-amino-6-aryl- (I, $R' =$ aryl, $R'' = NH_2$)[9] and 6:7-dialkyl- (I, $R' = R'' =$ alkyl)[5,6]. As a further simplification of structure we first made 2:4-diamino-5-4′-chlorophenylazo-6-dimethylaminopyrimidine (II, R = Cl) (No. 24) since this would exist in the *trans* form[10] (as shown) and would therefore show some spacial similarity to a 2:4-diamino-6-chlorophenylpteridine. The chlorine

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† The term antifolic acid is used to refer to an antagonist of any vitamin of the folic acid group.

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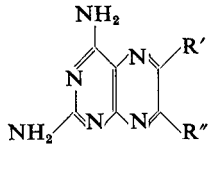
substituent in the benzene ring was selected in the hope that it would have a favourable effect because of the marked enhancement of antifolic activity in the 4:6-diamino-1-aryl-1:2-dihydro-5-triazines by the introduction of halogen substituents in position 3′ or 4′ or 3′ and 4′ of the benzene ring[11]. The discovery of antifolic activity in the azo-pyrimidine (II, R = Cl) encouraged further exploration using a diethanolamino- and morpholino-, instead of the dimethylamino- substituent but when it was found that much greater activity arose from using an unsubstituted amino group the series III was explored in detail. In the pyrimidine ring, variations involved the hydroxy, amino, thio, chloro and 4′-chlorophenylthio substituents and in the benzene ring chloro, bromo, nitro, methoxy and ethoxy substituents were used. In place of the benzene ring naphthalene and quinoline were introduced. Since amino-aryl-azopyrimidines (III) are, in general, easily oxidised to 8-azapurines[12] (IV) it was thought that this conversion might occur *in vivo* and that the 8-azapurines might therefore be active. Many active compounds were found in this series but the evidence, given later in this paper in the discussion of structure-activity relations, is against the hypothesis that the aryl-azopyrimidines owe their activity to this conversion. The 8-aryl-8-azapurines were all made by a general method[12] from the aryl-azopyrimidines (III) which were usually synthesised by the method[12] of coupling the appropriate pyrimidine and aryl diazonium salt. The azo compounds were oxidised by copper sulphate in boiling pyridine, the yields being greatly improved by passing oxygen through the reaction mixture. Azo compounds containing a tertiary amino group in the 4- (or 6-) position of the pyrimidine, e.g., II, were made by reacting the appropriate chloro-aryl-azopyrimidine (X) and secondary amine.

The dimethylamino derivatives (II, R = Cl and NO_2) were also formed by reacting the chloro-pyrimidines (X, R′ = Cl and NO_2, R″ = Cl) and guanidine in a mixture of ethanol and dimethylformamide, respectively under hot and cold conditions. Presumably the reaction of dimethylformamide and guanidine liberates dimethylamine. From X, (R′ = NO_2, R″ = Cl) in this reaction there is also formed 2:4-diamino-6-ethoxy-5(4′-nitrophenylazo)pyrimidine (X, R′ = NO_2, R″ = OEt) but none of the corresponding ethoxy derivative could be isolated from the chlorophenyl analogue (X, R′ = Cl, R″ = Cl). From X, (R′ = Cl, R″ = Cl) and hot 2-ethoxyethanol in the presence of guanidine the corresponding 2-ethoxyethoxy derivative (X, R′ = Cl, R″ = OCH_2CH_2OEt) was analogously formed, the guanidine being a sufficiently strong base to take the place of the sodium alkoxide usually used in this type of reaction. When 4:6-diamino-2-thiopyrimidine was coupled with *p*-chlorobenzene diazonium chloride the desired 4:6-diamino-5(4′-chlorophenylazo)-2-thiopyrimidine (XI, R = SH) was formed in small yield, the major product (XI, R = SC_6H_4Cl (*p*)) having been formed by coupling with two mols of the diazonium chloride. Previously[13] 2-thiouracil and 4-methyl-2-thiouracil have been coupled with this diazonium chloride but yielded only the product derived from two mols of the reagent; in our case the 4:6-diamino groups therefore appear to direct the coupling to the 5-position more strongly.

In order to observe the effect of changing the position of the aryl substituent in the 8-azapurine series some 9-aryl derivatives were made.

9-Phenyl-2-amino-8-azapurine (VII, R′ = H, R″ = Ph) was synthesised from 2-amino-4-chloro-5-nitropyrimidine by condensation with aniline to form 2-amino-4-anilino-5-nitropyrimidine, reduction, and ring-closure of the 4 : 5-diamine by treatment with nitrous acid. Similarly were prepared the *p*-chlorophenyl analogue (VII, R′ = H, R″ = $C_6H_4Cl(p)$) and the 6-methyl derivative (VII, R′ = CH_3, R″ = Ph).



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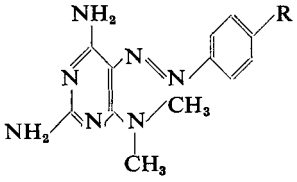

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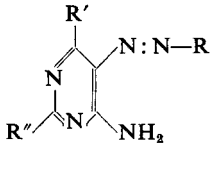

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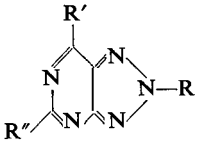

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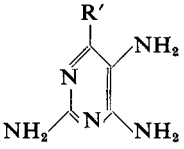

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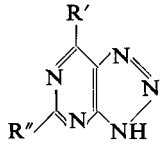

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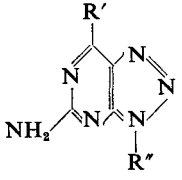

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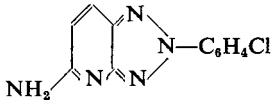

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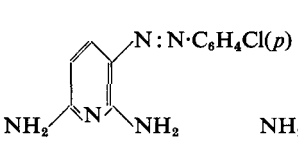

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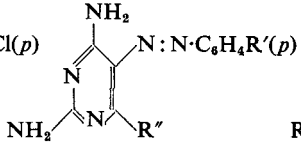

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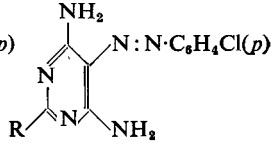

(XI)

From structural analogy, the two pyridine-containing structures VIII and IX were potentially interesting especially since the benzeneazo analogue of IX (2-benzeneazo-2 : 6-diaminopyridine, "Pyridium") is a powerful bactericide. These compounds (Nos. 66 and 60 respectively), which were synthesised by methods exactly analogous to those used for the azopyrimidines and 8-aryl-8-azapurines, were inactive.

6-Thio-8-aryl-8-azapurines were made by thiation of the 6-hydroxy analogues with phosphorus pentasulphide in boiling pyridine solution. 2 : 6-Diamino-8-azapurine (VI, R′ = R″ = NH_2) (No. 65) made according

to Cavalieri and others[14], and 8-azapurine (No. 64), made by ring-closure of 4:5-diaminopyrimidine, were synthesised to ascertain the effect on activity of removing successively the aryl substituent and the amino groups from the diamino-8-azapurines. Both were inactive.

2:4:5-Triamino-6-hydroxypyrimidine (No. 68) and 2:4:5:6-tetraminopyrimidine (No. 67) were made, by Traube's method, for antifolic tests as possible *in vivo* reduction products of the diamino-hydroxy and triamino-arylazopyrimidines respectively. Since both types of azopyrimidine are active, the fact that V ($R' = OH$)[15] and V ($R' = NH_2$)[16] are respectively inactive and active, argues against the hypothesis that they might be the active forms.

New compounds are indicated in the experimental section by italics and, in the Tables, by the absence of references to their preparation.

Experimental

Melting points were determined using an electrically heated copper block and are uncorrected unless otherwise stated. Samples for analysis were dried at 100° in a high vacuum unless otherwise stated. Analyses were by Drs. Weiler and Strauss (Oxford) and by Mr. P. R. W. Baker (Beckenham).

2:4-*Diamino*-6-*chloro*-5(4′-*nitrophenylazo*)-*pyrimidine* (CB. 2297, No. 19)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was cooled in ice-water and stirred and then treated with a solution of *p*-nitrobenzenediazonium chloride (from *p*-nitraniline (1·38 g.)), freed from nitrous acid by treatment with excess of sulphamic acid. A slight change in colour was noted. After stirring the solution for 5 minutes, it was treated with crystalline sodium acetate to bring the pH to 6–7 and stirring was continued for 5 hours, during which time a thick orange-red precipitate was obtained. This was collected, washed with water copiously, dried and recrystallised from aqueous 2-ethoxyethanol to yield the *azo-compound* as scarlet needles, m.p. 298 to 299° (decomp.) (Found: C, 38·7; H, 3·3; N, 31·4; Cl, 11·3. $C_{10}H_8O_2N_7Cl.1H_2O$ requires C, 38·5; H, 3·2; N, 31·5; Cl, 11·4. Found on a sample dried at 110°; C, 41·15; H, 2·9; loss 5·8. $C_{10}H_8O_2N_7Cl$ requires C, 40·9; H, 2·7; loss, 5·8 per cent).

2:4-*Diamino*-6-*chloro*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2298, No. 20)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was treated with *p*-chlorobenzenediazonium chloride (from *p*-chloroaniline (1·275 g.)) and the product isolated as above. Recrystallised from aqueous 2-ethoxyethanol, the *azo-compound* formed fine yellow needles m.p. 271 to 272° (decomp.). (Found: C, 42·1; H, 3·1; N, 29·3; Cl, 25·0. $C_{10}H_8N_6Cl_2$ requires C, 42·4; H, 2·8; N, 29·7; Cl, 25·1 per cent).

2:4-*Diamino*-6-*dimethylamino*-5(4′-*nitrophenylazo*)-*pyrimidine* (CB. 2269, No. 21)

Method 1. 2:4-Diamino-6-chloro-5(4′-nitrophenylazo)-pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was treated with an ethanolic solution

of dimethylamine (33 per cent, 3 ml.) and the mixture was heated under reflux at 100° when the azo compound passed into solution. After one hour, water was added to turbidity and the solution filtered. On cooling, the solution deposited *dark maroon crystals* with a green lustre (0·87 g.), which after recrystallisation from aqueous 2-ethoxyethanol had m.p. 252 to 255°, undepressed on admixture with a sample prepared according to Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4′-nitrophenylazo)pyrimidine (1 g.) was dissolved in warm dimethylformamide (20 ml.) and treated with a solution of guanidine (prepared from guanidine hydrochloride (0·95 g.) and sodium (0·23 g.) dissolved in ethanol (20 ml.), with removal of the precipitated sodium chloride). There was an immediate precipitation of crimson solid, which was allowed to stand overnight. The precipitate (1 g.) was then collected and fractionally crystallised from aqueous 2-ethoxyethanol to yield:—

(i) 2:4-*Diamino*-6-*dimethylamino*-5(4′-*nitrophenylazo*)*pyrimidine*. This formed the less soluble fraction and recrystallised from aqueous 2-ethoxyethanol as deep maroon leaflets with a strong metallic green lustre, m.p. 252 to 255°. (Found: C, 48·0; H, 4·6; N, 37·45; $C_{12}H_{14}O_2N_8$ requires C, 47·7; H, 4·6; N, 37·1 per cent). This was sparingly soluble in dilute hydrochloric acid and on examination in ultra-violet light, the solution had a light blue fluorescence.

(ii) 2:4-*Diamino*-6-*ethoxy*-5(4′-*nitrophenylazo*)*pyrimidine*. This was precipitated on dilution of the mother liquors from which (i) had separated. It recrystallised from aqueous 2-ethoxyethanol as deep orange needles, m.p. 214 to 215°. (Found: C, 47·7; H, 4·6; N, 32·0. $C_{12}H_{13}O_3N_7$ requires C, 47·5; H, 4·3; N, 32·35 per cent). It yielded a sparingly soluble yellow hydrochloride with dilute hydrochloric acid.

2:4-*Diamino*-6-*morpholino*-5(4′-*nitrophenylazo*)*pyrimidine* (CB. 2270, No. 22)

2:4-Diamino-6-chloro-5(4′-nitrophenylazo)pyrimidine (1 g.) was treated with dry morpholine (10 ml.) when the mixture became warm. The reaction was completed by heating under reflux for 10 minutes, and on cooling the mixture deposited red crystals. These were collected, washed with cold ethanol and recrystallised from aqueous 2-ethoxyethanol to yield the *morpholino* compound as deep red prisms, m.p. 275° (decomp.). (Found: C, 49·2; H, 4·60; N, 32·5. $C_{14}H_{16}O_3N_8$ requires C, 48·9; H, 4·65; N, 32·55 per cent). The compound had a pronounced golden-green metallic lustre.

2:4-*Diamino*-6-*diethanolamino*-5(4′-*nitrophenylazo*)*pyrimidine* (CB. 2271, No. 23)

This was prepared similarly from the azo compound (1 g.) and dry diethanolamine (5 ml.). The *diethanolamino* compound, obtained by dilution of the reaction mixture with water, crystallised from very dilute ethanol as deep maroon plates, with a metallic lustre, m.p. 211 to 212°

(decomp.). (Found: C, 46·2; H, 5·1; N, 30·7. $C_{14}H_{18}O_4N_8$ requires C, 46·4; H, 5·0; N, 30·9 per cent). It was very soluble in ethanol.

2:4-*Diamino*-5(4′-*chlorophenylazo*)-6-*dimethylamino pyrimidine* (CB. 2266, No. 24)

Method 1. 2:4-Diamino-6-chloro-5(4′-chlorophenylazo) pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was heated under reflux for one hour with an ethanolic solution of dimethylamine (33 per cent, 3 ml.). The clear solution was then filtered and concentrated, and on dilution then yielded a yellow precipitate. This on recrystallisation from aqueous 2-ethoxyethanol yielded the *dimethylamino* compound (0·6 g.) as long lustrous yellow needles, m.p. 210° (corr.), undepressed on admixture with the product from Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4′-chlorophenylazo)pyrimidine (0·5 g.) was dissolved in dimethylformamide (10 ml.) and treated with a solution of guanidine in ethanol (from guanidine hydrochloride (0·675 g.) and a solution of sodium (0·16 g.) in ethanol (5 ml.), with removal of sodium chloride). The mixture was heated under reflux at 60 to 70° for 4 hours and then the solvent was removed under reduced pressure. Addition of water yielded a yellow solid which was crystallised from aqueous 2-ethoxyethanol to yield the 6-*dimethylamino* compound as yellow needles, m.p. 210° (corr.) or lustrous golden leaflets, m.p. 187 to 188° depending on the rate of cooling. (Found: C, 49·3; H, 4·9; N, 33·75; Cl, 12·3. $C_{12}H_{14}N_7Cl$ requires C, 49·4; H, 4·8; N, 33·6; Cl, 12·2 per cent).

Careful search failed to reveal a second component (cf. above).

2:4-*Diamino*-5(4′-*chlorophenylazo*-6(*β-ethoxyethoxy*))*pyrimidine*

2:4-Diamino-6-chloro-5(4′-chlorophenylazo)pyrimidine (0·5 g.) was added to a solution of guanidine (from guanidine hydrochloride (0·675 g.) and sodium (0·16 g.) dissolved in 2-ethoxyethanol (20 ml.) with removal of the sodium chloride) and the mixture heated under reflux at 80° for three hours. Concentration of the filtered solution under reduced pressure at 100° and dilution of the residue with water yielded a yellow solid which on recrystallisation from aqueous 2-ethoxy ethanol (90 per cent) afforded the *azopyrimidine* as yellow lustrous silky needles, m.p. 161 to 162°. (Found: C, 50·1; H, 5·0; N, 25·1; Cl, 10·6. $C_{14}H_{17}O_2N_6Cl$ requires C, 49·95; H, 5·05; N, 25·0; Cl, 10·55 per cent).

2:4-*Diamino*-5(4′-*chlorophenylazo*)-6-*morpholinopyrimidine* (CB. 2279, No. 25)

The chlorophenylazo-6-chloropyrimidine (0·3 g.) and morpholine (2 ml.) were heated together under reflux for one hour, cooled and diluted with water to yield a yellow precipitate. This, on crystallisation from aqueous 2-ethoxyethanol, afforded the *morpholino pyrimidine* as golden-yellow lustrous plates, m.p. 221 to 222°. (Found: C, 50·7; H, 4·7; N, 28·9; Cl, 10·5. $C_{14}H_{16}ON_7Cl$ requires C, 50·4; H, 4·8; N, 29·4; Cl, 10·65 per cent).

2:4-*Diamino*-5(4'-*chlorophenylazo*)-6-*diethanolaminopyrimidine* (CB. 2280, No. 26)

The chlorophenylazo-6-chloropyrimidine (1·0 g.) and diethanolamine (5 ml.) were heated together at 140° for 2 hours, cooled and diluted with water. The resulting oily gum crystallised on being allowed to stand overnight and the solid, after crystallisation from very dilute ethanol, yielded the *diethanolamino* compound as orange brown prisms, m.p. 160 to 161°. (Found: C, 48·15; H, 5·12; N, 28·25; Cl, 10·4. $C_{14}H_{18}O_2N_7Cl$ requires C, 47·8; H, 5·12; N, 27·9; Cl, 10·1 per cent).

2:4:6-*Triamino*-5-*arylazopyrimidines*

General Method. 2:4:6-Triaminopyrimidine (5·0 g., 1/25 mol.) was dissolved in water (160 ml.) containing crystalline sodium acetate (16 g.) and the mixture stirred mechanically at 0° during the addition of a filtered solution of the required diazotised amine. The latter solution was generally prepared by dissolving the arylamine (1/25 mol.) in a mixture of concentrated hydrochloric acid (12 ml.) and water (20 ml.), cooling to 0° and adding a solution of sodium nitrite (3·04 g.) in water (20 ml.); after 2–3 minutes, an excess of solid sulphamic acid was added and the solution filtered rapidly through a chilled funnel under suction. Modification of the procedure was necessary only if the amine was too weakly basic to be soluble in dilute hydrochloric acid. An extreme example was 2:4:6-tribromoaniline, which was diazotised as follows. The amine (6·6 g., 1/50 mol.) was dissolved in hot glacial acetic acid (79 ml.) and crystallised on cooling. The suspension of small crystals was added to a solution of sodium nitrite (1·54 g.) in cold concentrated sulphuric acid (10 ml.) and gave a pale yellow solution. This coupled normally on addition to a solution of triaminopyrimidine (2·5 g.) in water (500 ml.) and the product was collected after adjustment of the pH of the solution to 6–7 by the addition of sodium acetate crystals.

Occasionally, despite the presence of sodium acetate in the solution before addition of the diazonium salt, the azo compound was precipitated as the hydrochloride. This could be converted to the base by boiling with aqueous pyridine.

The azo compounds so obtained are listed in Table I.

Other 4-*amino*-5-*arylazopyrimidines*

2:4-*Diamino*-5(4'-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2288, No. 27)

2:4-Diamino-6-hydroxypyrimidine hydrochloride (6·5 g.) was suspended in water (360 ml.) stirred with sodium acetate (42 g.) and filtered. A solution of *p*-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) was slowly added to the ice-cold filtrate with stirring and the orange-yellow precipitate was collected after two hours. It was crystallised from aqueous pyridine when a pyridine salt was obtained, which was decomposed by stirring with hot ethanol to liberate the *azo-compound* as a yellow microcrystalline powder, m.p. 314 to 315° (decomp.). (Found:

TABLE I

ARYL-AZOPYRIMIDINES (III, $R' = R'' = NH_2$)

CB. No.	R	Formula	Colour and crystal form	M.p. °	Solvent‡	Analysis									
						Found					Required				
						C	H	N	Cl	Br	C	H	N	Cl	Br
*2295 No. 1	C_6H_5–	$C_{10}H_{11}N_7$	Yellow leaflets	262–263	CW	52·5	4·6	42·4	—	—	52·4	4·8	42·8	—	—
2310 No. 2	2-ClC_6H_4–	$C_{10}H_{10}N_7Cl$	Yellow needles	290–291	PW	45·7	3·9	36·6	13·6	—	45·5	3·8	37·2	13·5	—
2309 No. 3	3-ClC_6H_4–	,,	Yellow needles	264–265	PW	45·7	3·7	37·0	13·6	—	,,	,,	,,	,,	—
2277 No. 4	4-ClC_6H_4–	,,	Golden-yellow flat needles	262	CW	45·9	4·2	36·7	13·3	—	,,	,,	,,	,,	—
2328 No. 5	2-BrC_6H_4–	$C_{10}H_{10}N_7Br$	Golden-yellow crystals	298–299	CW	39·5	3·3	31·9	—	26·0	39·0	3·25	31·8	—	26·0
2311 No. 6	4-BrC_6H_4–	,,	Yellow prisms	261–262	CW	39·0	3·3	31·7	—	25·7	,,	,,	,,	—	,,
2326 No. 7	2:3-$Cl_2C_6H_3$–	$C_{10}H_9N_7Cl_2$	Orange-yellow leaflets	316–317	CW	40·6	2·9	32·7	23·9	—	40·3	3·0	32·9	23·8	—
2307 No. 8	2:4-$Cl_2C_6H_3$–	,,	Long yellow needles	304–305 (decomp.)	PW	40·3	3·0	32·5	24·1	—	,,	,,	,,	,,	—
2322 No. 9	2:5-$Cl_2C_6H_3$–	,,	Yellow needles	319–320	PW	40·4	3·0	32·7	23·6	—	,,	,,	,,	,,	—
2283 No. 10	3:4-$Cl_2C_6H_3$–	,,	Yellow leaflets	269–270	PW	40·4	3·0	32·6	23·7	—	,,	,,	,,	,,	—
2349 No. 11	2:4-$Br_2C_6H_3$–	$C_{10}H_9N_7Br_2$	Orange-yellow leaflets	328–329	PW	31·3	2·5	25·3	—	41·3	31·0	2·3	25·3	—	41·4
2347 No. 12	2:4:6-$Br_3C_6H_2$–	$C_{10}H_8N_7Br_3$	Orange-yellow needles	304–305 (decomp.)	CW	25·8	2·0	20·6	—	51·4	25·75	1·7	21·05	—	51·5
2299 No. 13	4-$MeOC_6H_4$–	$C_{11}H_{13}ON_7$	Orange-yellow prisms	231–232	CW	50·8	5·4	37·7	—	—	51·0	5·0	37·8	—	—
2323 No. 14	4-$EtOC_6H_4$–	$C_{12}H_{15}ON_7$	Yellow needles	280–282	CW	53·1	5·3	35·7	—	—	52·8	5·5	35·9	—	—
†2296 No. 15	4-$NO_2C_6H_4$–	$C_{10}H_{10}O_2N_8$	Red needles	352–353	ACOH	44·1	3·5	40·7			43·8	3·65	40·9	—	—
2330 No. 16	1-naphthyl	$C_{14}H_{13}N_7$	Golden-brown plates	264–265 (decomp.)	CW	60·6	4·6	35·3	—	—	60·2	4·7	35·15	—	—
2315 No. 17	2-naphthyl	,,	Yellow needles	298 (decomp.)	B	59·9	4·4	34·9	—	—	,,	,,	,,	—	—
2331 No. 18	3-quinolyl	$C_{13}H_{12}N_8$	Orange leaflets, blue lustre	315–316 (decomp.)	PW	56·0	4·5	39·9	—	—	55·7	4·3	40·0	—	—

* First prepared by Hartzel and Benson(*J. Amer. chem. Soc.*, 1954, 76, 2263) who give m.p. 235° (decomp.).
† Yellow acetate, initially isolated, is decomposed either on standing or on washing with ethanol and ether.
‡ Solvents: B, *n*-butanol; CW, aqueous 2-ethoxyethanol; PW, aqueous pyridine; ACOH, glacial acetic acid.

C, 45·5; H, 3·5; N, 31·5; Cl, 13·2. $C_{10}H_9ON_6Cl$ requires C, 45·4; H, 3·4; N, 31·73; Cl, 13·4 per cent).

2:4-*Diamino*-5(4′-*bromophenylazo*)-6-*hydroxypyrimidine* (CB. 2336, No. 28)

This was obtained in a similar manner when *p*-bromobenzene-diazonium chloride was used. The *azo-compound* was purified by successive crystallisations from aqueous pyridine and aqueous 2-ethoxyethanol and formed yellow crystals. (Found: C, 38·9; H, 3·5; N, 27·3; Br, 26·0. $C_{10}H_9ON_6Br$ requires C, 38·9; H, 3·0; N, 27·2; Br, 25·9 per cent).

4-*Amino*-5(4′-*chlorophenylazo*)-2:6-*dihydroxypyrimidine* (CB. 2313, No. 29)

4-Amino-2:6-dihydroxypyrimidine (5·1 g.) was suspended in water and sufficient 2N sodium hydroxide was added dropwise to yield a clear solution. This solution was then cooled in ice, stirred and treated with a filtered solution of *p*-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)). Glacial acetic acid was added dropwise to the mixture to adjust the pH to 5–6 and after the mixture had remained at 0° in the ice-chest overnight, the product was collected. It was purified by crystallisation from hot 90 per cent formic acid by the addition of water and the resulting yellow solid was dissolved in hot 6N ammonium hydroxide, the solution filtered and the product reprecipitated by neutralisation using glacial acetic acetic acid. The *azo-compound* formed a fine yellow powder which was thoroughly washed by means of water, ethanol and ether successively. (Found: C, 45·15; H, 2·9; N, 26·2; Cl, 13·2; $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2:4-*Diamino*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2329, No. 30)

This was prepared as described by Brown[17]. It formed yellow needles from aqueous 2-ethoxyethanol, m.p. 282 to 283° (decomp.). Brown (*loc. cit.*) gives m.p. 271 to 272°.

4-*Amino*-5(4′-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2304, No. 31)

A solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (10·2 g.)) was added slowly with stirring to a filtered, ice-cold solution of 4-amino-6-hydroxypyrimidine (8·82 g.) in water (850 ml.). The pH of the reaction mixture was adjusted to 5–6 by the addition of crystalline sodium acetate and the product collected after a period. Recrystallisation from aqueous 2-ethoxyethanol afforded the *azo-compound* as orange needles, m.p. 300°. (Found: C, 48·6; H, 3·3; N, 27·8; Cl, 14·1. $C_{10}H_8ON_5Cl$ requires C, 48·1; H, 3·2; N, 28·1; Cl, 14·2 per cent).

4:6-*Diamino*-5(4′-*chlorophenylazo*)-2-*thiopyrimidine* (CB. 2292, No. 32) and 4:6-*Diamino*-5(4′-*chlorophenylazo*)-2(4′-*chlorophenylthio*) *pyrimidine* (CB. 2290, No. 33)

A solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (5·1 g.)) was added slowly to an ice-cold, stirred solution of 4:6-diamino-2-thiopyrimidine (5·68 g.) in dilute hydrochloric acid (0·1N, 500 ml.).

On adjusting the pH to 5–6 by the addition of crystalline sodium acetate, a slow precipitation of yellow solid was observed. This was completed after the mixture had stood overnight at 0°. The product was extracted with boiling ethanol and the hot filtrate diluted with water to yield 4:6-*diamino*-5(4′-*chlorophenylazo*)-2-(4′-*chlorophenylthio*) *pyrimidine* as yellow feathery needles from aqueous ethanol, m.p. 211 to 212°. (Found: C, 49·3; H, 3·0; N, 21·35; S, 8·35; Cl, 17·6. $C_{16}H_{12}N_6SCl_2$ requires C, 49·1; H, 3·1; N, 21·5; S, 8·2; Cl, 18·2 per cent).

The residue from the ethanol extraction was crystallised several times from aqueous pyridine and afforded 4:6-diamino-5 (4′-chlorophenylazo)-2-thiopyrimidine as yellow needles, m.p. 278 to 279° (decomp.). Found: C, 43·2; H, 3·1; N, 29·7; S, 11·3; Cl, 13·0. $C_{10}H_9N_6SCl$ requires C, 42·8; H, 3·2; N, 30·0; S, 11·4; Cl, 12·7 per cent).

4:6-*Diamino*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2287, No. 34)·

4:6-Diamino pyrimidine (4·4 g.) was dissolved in dilute hydrochloric acid (500 ml. 0·04N) and coupled by treating it with a filtered solution of 4-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) in the usual manner. The addition of solid sodium acetate to the reaction mixture afforded a precipitate which was collected and recrystallised from aqueous 2-ethoxyethanol when the azo compound was obtained as flat yellow needles, m.p. 299 to 300° (decomp.). (Found: C, 48·6; H, 3·5; N, 34·0; Cl, 14·25. Calc. for $C_{10}H_9N_6Cl$: C, 48·3; H, 3·6; N, 33·85; Cl, 14·3 per cent). Lythgoe, Todd and Topham[18] give m.p. 301 to 302° (decomp.).

The preparation of 2:6-diamino-3(4′-chlorophenylazo)pyridine is given at the end of the experimental section.

8-*Aryl*-8-*azapurines*

With the exception of the few members noted below, which include a 1-deaza-8-azapurine (No. 60), the compounds of this series were all prepared in the following general manner.

General Method of preparation of 8-*aryl-azapurines*

The corresponding azopyrimidine (4 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate (10 g.). When the azo compound was insoluble in this mixture either a larger proportion of pyridine was used, e.g., 200 ml. pyridine to 100 ml. of water, or else a larger volume of mixed solvents in 1:1 proportion. The mixture was placed in a three-necked flask equipped with reflux condenser and a gas inlet tube reaching to the bottom of the flask, and heated to boiling under reflux. A slow stream of oxygen from a cylinder was then passed until the reaction was complete, which was indicated when the solution changed from a greenish-yellow hue to a rich royal blue colour. The hot solution was then poured into water (2 litres) and allowed to stand overnight to ensure complete precipitation. The 8-azapurine was then collected at the pump, washed with copious quantities of water and then with ethanol and finally ether. In nearly every case the yield was quantitative.

TABLE II

8-ARYL-8-AZAPURINES (IV)

CB No.	R	R′	R″	Formula	M.p. °	Solvent§	Analysis											
							Found						Required					
							C	H	N	Cl	Br	S	C	H	N	Cl	Br	S
*†2314 No. 35	C_6H_5–	NH_2	NH_2	$C_{10}H_9N_7$	344–345 (decomp.)	20 F	—	—	—	—	—	—	—	—	—	—	—	—
2321 No. 36	2-ClC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Cl$	283–284	20 F	45·9	3·2	37·7	13·3	—	—	45·9	3·1	37·5	13·6	—	—
2308 No. 37	3-ClC_6H_4–	NH_2	NH_2	,,	350 (decomp.)	20 F	46·2	3·0	37·2	13·8	—	—	,,	,,	,,	,,	—	—
2278 No. 38	4-ClC_6H_4–	NH_2	NH_2	,,	>360	20 F	46·3	2·9	37·3	13·4	—	—	,,	,,	,,	,,	—	—
2332 No. 39	2-BrC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Br$	271–272	10 F	37·65	2·8	27·5	—	23·2	—	37·5	2·8	27·85	—	22·7	—
2319 No. 40	4-BrC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Br + \frac{1}{2}HCO_2H$	>360	20 F	38·1	3·0	29·8	—	24·5	—	38·3	2·7	29·8	—	24·3	—
2333 No. 41	2:3-$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2 + \frac{1}{2}HCO_2H$	287–288	20 F	39·35	2·5	30·6	22·7	—	—	39·5	2·5	30·7	22·3	—	—
2318 No. 42	2:4-$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	320–321	20 F	40·1	2·4	32·5	23·85	—	—	40·55	2·4	33·1	24·0	—	—
2324 No. 43	2:5-$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	312–313	20 F	40·7	2·5	32·45	23·8	—	—	40·55	2·4	33·1	24·0	—	—
2282 No. 44	3:4-$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	>360	20 F	40·0	2·5	32·9	24·2	—	—	40·55	2·4	33·1	24·0	—	—
2364 No. 45	2:4-$Br_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Br_2 + HCO_2H$	279–281 (decomp.)	20 F	30·6	2·5	22·5	—	37·0	—	30·65	2·1	22·75	—	37·15	—
2350 No. 46	2:4:6-$Br_3C_6H_2$	NH_2	NH_2	$C_{10}H_6N_7Br_3$	286–287	AW	26·2	1·3	20·9	—	51·3	—	25·9	1·3	21·1	—	51·7	—
2327 No. 47	4-EtO-C_6H_4–	NH_2	NH_2	$C_{12}H_{13}ON_7 + \frac{1}{4}HCO_2H$	310–311	20 F	51·7	4·4	34·5	—	—	—	52·0	4·8	34·7	—	—	—
2335 No. 48	1-$C_{10}H_7$–	NH_2	NH_2	$C_{14}H_{11}N_7$	290–291	20 F	60·4	3·7	35·2	—	—	—	60·7	3·9	35·4	—	—	—
‡2289 No. 49	4-ClC_6H_4–	OH	NH_2	$C_{10}H_7ON_6Cl$	>360	80 F	45·7	2·7	31·9	13·2	—	—	45·7	2·7	32·2	13·5	—	—
2337 No. 50	4-BrC_6H_4–	OH	NH_2	$C_{10}H_7ON_6Br$	>360	80 F	39·0	2·2	27·3	—	26·6	—	39·1	2·3	27·4	—	26·1	—
2285 No. 51	4-ClC_6H_4–	NMe_2	NH_2	$C_{12}H_{12}N_7Cl.HCl$	287–288	2N HCl	44·5	3·8	29·9	21·6	—	—	44·2	3·9	30·05	21·8	—	—
2293 No. 52	4-$NO_2C_6H_4$–	NMe_2	NH_2	$C_{12}H_{12}O_2N_8$	316–318	DMF	48·1	3·9	37·0	—	—	—	48·0	4·0	37·3	—	—	—
2291 No. 53	4-ClC_6H_4–	NH_2	4-ClC_6H_3S	$C_{16}H_{10}N_6SCl_2$	325–326 (decomp.)	80 F	49·3	2·7	21·25	17·9	—	8·1	49·4	2·6	21·6	18·3	—	8·2
2312 No. 54	4-ClC_6H_4–	NH_2	H	$C_{10}H_7N_6Cl$	367 (decomp.)	ACOH	49·1	3·1	34·0	14·1	—	—	48·7	2·8	34·1	14·4	—	—
2305 No. 55	4-ClC_6H_4–	OH	H	$C_{10}H_6ON_6Cl$	339–340	CW	48·7	2·5	28·5	14·8	—	—	48·5	2·4	28·3	14·3	—	—

* Parker and Webb, U.S.P. 2,543,333; *Chem. Abstr.*, 1951, **45**, 7605.
† Hartzel and Benson (*J. Amer. chem. Soc.*, 1954, **76**, 2263) give m.p. >300°.
‡ No preparation recorded in literature; compound mentioned in Stock and others (*Cancer Research, Suppl.* 1, p. 104 as compound No. 1189 (m.p. 375°), obtained from Calco Division, American Cyanamid Co.
§ Solvents: F, Aqueous formic acid, percentage content indicated; DMF, Dimethylformamide; ACOH, Glacial acetic acid; CW, Aqueous 2-ethoxyethanol; AW, Aqueous ethanol.

Table II lists the compounds prepared in this way. All were colourless solids, generally of microcrystalline form. The majority could only be crystallised from aqueous formic acid and occasionally retained formic acid tenaciously after prolonged drying at 100°. In every case the acid content was confirmed by drying at a higher temperature and recording the required loss, the product then analysed for carbon and hydrogen with satisfactory results. It is not possible to show this in the Table.

8-(4′-*Chlorophenyl*)-6-*thio*-8-*azapurine* (CB. 2320, No. 56)

8-(4′-Chlorophenyl)6-hydroxy-8-azapurine (3 g.) in dry pyridine (50 ml.) was treated with phosphorus pentasulphide (3 g.) and the mixture was heated under reflux for one and a half hours, all material dissolving during the initial half hour to yield a dark-brown solution. The reaction mixture was allowed to cool somewhat and was then smoothly decomposed by adding it to hot water (100 ml.). The product (2·87 g.) was collected next day and washed thoroughly with water and then with ethanol and ether. Two crystallisations from aqueous pyridine (charcoal) afforded the *thio-compound* as pale golden lustrous needles, m.p. 357 to 358° (decomp.). (Found: C, 46·0; H, 2·5; N, 26·3; S, 12·1; Cl, 13·4. $C_{10}H_6N_5SCl$ requires C, 45·5; H, 2·3; N, 26·6; S, 12·15; Cl, 13·5 per cent).

2-*Amino*-8(4′-*Chlorophenyl*)-6-*thio*-8-*azapurine* (CB. 2294, No. 57)

A mixture of 2-amino-8(4′-chlorophenyl)-6-hydroxy-8-azapurine (1 g.) and phosphorus pentasulphide (1 g.) in dry pyridine (50 ml.) was heated under reflux for two hours. A further portion of phosphorus pentasulphide (2 g.) and pyridine (50 ml.) was then added and heating continued. All material had dissolved after a further hour and after a total time of reflux of 4 hours, the mixture was decomposed by pouring into hot water (200 ml.). The primrose-yellow precipitate (Crude 0·91 g.) which was formed was collected next day and crystallised from pyridine (charcoal) to yield the *thio compound* as a yellow powder, m.p. 362 to 363° (decomp.). (Found: C, 45·3; H, 3·0; N, 28·9; S, 10·4; Cl, 12·1. $C_{10}H_7N_6SCl$–1/4 C_5H_5N requires C, 45·3; H, 2·8; N, 29·3; S, 10·7; Cl, 11·9 per cent).

2-*Amino*-6-*hydroxy*-8(3′-*quinolyl*)-8-*azapurine* (CB. 2338, No. 58)

2:4:6-Triamino-5(3′-quinolylazo) pyrimidine (12·0 g.) in pyridine (600 ml.) and water (100 ml.) was treated with a solution of copper sulphate pentahydrate (40 g.) in hot water (100 ml.) and the clear solution was heated in a three-necked flask fitted with reflux condenser while a slow stream of oxygen was passed through the liquid. A creamish-yellow precipitate soon began to form, and after two hours the colour of the supernatant solution assumed a clear royal blue. Whereupon the mixture was poured into water (4 l.). The product was collected next day, and purified by solution in hot 2N hydrochloric acid, treatment with charcoal and reprecipitation with 2N ammonium hydroxide several times. The product formed a *pale cream solid*, m.p. $>$ 360°. Found: C, 56·0; H, 3·2; N, 35·2; $C_{13}H_9ON_7$ requires C, 56·0; H, 3·2; N, 35·15 per cent).

Besides being soluble in dilute acid, the compound was soluble in hot dilute sodium hydroxide, revealing the presence of a hydroxy group.

2:6-*Diamino*-8-(3′-*quinolyl*)-8-*azapurine* (CB. 2381, No. 59)

The ring closure of triamino-5(3′-quinolylazo)pyrimidine was carried out exactly as described above. The crude product was recrystallised from propylene glycol (charcoal) several times, washed thoroughly by trituration with ethanol and finally ether and yielded a *pale cream solid*, m.p. > 360°, which however, was now not soluble in hot dilute sodium hydroxide. To obtain satisfactory analytical figures, it was necessary to dry the sample to constant weight at 140°. (Found: C, 56·3; H, 4·0; N, 39·9; $C_{13}H_{10}N_8$ requires C, 56·2; H, 3·6; N, 40·3 per cent).

5-*Amino*-2(4′-*chlorophenyl*)-*triazolo*(4′:5′-2:3)*pyridine* (CB. 2363, No. 60)

2:6-Diamino-3(4′-chlorophenylazo)pyridine (5 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate pentahydrate (10 g.) and was heated under reflux during the passage of oxygen. A rapid reaction occurred and a deep blue colour developed within 10 minutes. After one hour, the mixture was poured into water (2 l.) and the pale grey precipitate was collected. Crystallisation from aqueous 2-ethoxyethanol gave the *triazolopyridine* as long white felted needles, changing on standing to pale yellow prismatic needles. The change could be accelerated by warming; both forms melted at 258 to 259°. (Found: C, 54·1; H, 3·2; N, 28·65; Cl, 14·1. $C_{11}H_8N_5Cl$ requires C, 53·8; H, 3·3; N, 28·5; Cl, 14·5 per cent.) Treatment of this compound with hot acetic anhydride afforded the 5-*acetamido* derivative as colourless needles, m.p. 296 to 297° from *n*-butanol. (Found: C, 53·0; H, 3·5; N, 23·2; Cl, 12·2. $C_{13}H_{10}ON_5Cl.\frac{1}{2}H_2O$ requires C, 52·6; H, 3·5; N, 23·6; Cl, 12·0 per cent).

9-*Aryl*-8-*azapurines*

A. 2-*Amino*-9-*phenyl*-8-*azapurine*

(i) 2-*Amino*-4-*anilino*-5-*nitropyrimidine.* 4-Anilino-2-chloro-5-nitropyrimidine (3·0 g.)[19] was heated for four hours at 100° in a sealed tube with saturated ethanolic ammonia (25 ml.). The tube was cooled, opened and the lemon yellow product (2·91 g.) collected and crystallised from *n*-butanol to give the *aminonitropyrimidine* as lemon-yellow needles, m.p. 206 to 207°. (Found: C, 52·3; H, 3·9; N, 30·05. $C_{10}H_9O_2N_5$ requires C, 52·0; H, 3·9; N, 30·3 per cent).

(ii) 2:5-*Diamino*-4-*anilino pyrimidine.* The above aminonitropyrimidine (1·8 g.) was stirred into a solution of stannous chloride (7·6 g.) in concentrated hydrochloric acid (38 ml.) and the mixture was gently warmed on the steam bath. When all the yellow solid had been replaced by a white precipitate, the mixture was cooled and the solid collected and dissolved in water. The solution was treated with 40 per cent sodium hydroxide solution and the white precipitate collected using chloroform. Evaporation of the dried extract gave a pale yellow residue which was

crystallised from 2N hydrochloric acid (charcoal) to yield the *triamine dihydrochloride* as colourless needles, m.p. 249 to 250° (decomp.). (Found: 3, 44·0; H, 4·8; N, 25·9; Cl, 25·3. $C_{10}H_{11}N{\cdot}2HCl$ requires C, 43·8; H, 4·75; N, 25·6; Cl, 25·9 per cent). Treatment of an ethanolic solution of the dihydrochloride with an ethanolic solution of picric acid gave the *monopicrate* as yellow needles, m.p. 249 to 250° (decomp.) from aqueous ethanol. (Found: C, 44·4; H, 3·4; N, 25·6; $C_{10}H_{11}N_5{\cdot}C_6H_3O_7N_3$ requires C, 44·7; H, 3·3; N, 26·1 per cent).

(iii) 2-*Amino*-9-*phenyl*-8-*azapurine* (CB. 2355, No. 61). The above dihydrochloride (0·68 g.) was dissolved in water (40 ml.) containing a few drops of concentrated hydrochloric acid to suppress any hydrolysis and was stirred and cooled to 5°. A solution of sodium nitrite (0·24 g.) in water (1 ml.) was added when an immediate pale yellow precipitate was formed. After half an hour, the pH of the mixture was adjusted to 8 by the addition of ammonium hydroxide and the product was collected, and crystallised from aqueous methanol (charcoal). The *triazolopyrimidine* formed colourless rosettes of needles m.p. 167 to 168°. (Found: C, 56·6; H, 3·9; N, 39·7; $C_{10}H_8N_6$ requires C, 56·6; H, 3·8; N, 39·65 per cent).

B. 2-*Amino*-6-*methyl*-9-*phenyl*-8-*azapurine*

(i) 2-*Amino*-4-*anilino*-6-*methyl*-5-*nitropyrimidine*. 4-Anilino-2-chloro-6-methyl-5-nitropyrimidine (2·54 g.; Spickett[19], *loc. cit.*) was heated at 100° in a sealed tube with saturated methanolic ammonia solution (20 ml.) for 1½ hours. When cold, the tube was opened and the product (2·3 g.) was collected and crystallised from *n*-butanol to give the *amino* compound as yellow needles, m.p. 192 to 193°. (Found: C, 53·3; H, 4·3; N, 28·6. $C_{11}H_{11}O_2N_5$ requires C, 53·9; H, 4·5; N, 28·6 per cent).

(ii) 2:5-*Diamino*-4-*anilino*-6-*methylpyrimidine*. The nitro compound (3·0 g.) was reduced using a solution of stannous chloride (12 g.) in concentrated hydrochloric acid (60 ml.). The precipitated stannichloride was collected, decomposed with 40 per cent sodium hydroxide and the liberated base extracted with several portions of chloroform. Evaporation of the dried extracts gave the crude product as a crusty brownish solid (2·4 g.). This, on crystallisation from hot 2N hydrochloric acid (charcoal) gave colourless, lustrous needles, m.p. 239 to 240°, of the *dihydrochloride*. (Found: C, 45·8; H, 5·2; N, 24·45; Cl, 24·65. $C_{11}H_{13}N_5{\cdot}2HCl$ requires C, 45·9; H, 5·2; N, 24·3; Cl, 24·7 per cent). The *monopicrate* formed orange-yellow leaflets, m.p. 252 to 253° (decomp.) from dilute ethanol. (Found: C, 45·9; H, 3·8; N, 24·85. $C_{11}H_{13}N_5C_6H_3O_7N_3$ requires C, 45·95; H, 3·6; N, 25·25 per cent).

(iii) 2-*Amino*-6-*methyl*-9-*phenyl*-8-*azapurine* (CB. 2341, No. 62). 2,5-Diamino-4-anilino-6-methyl pyrimidine dihydrochloride (2·0 g.) in water (150 ml.) containing concentrated hydrochloric acid (4 drops) was cooled to 5° and treated with a solution of sodium nitrite (0·7 g.) in water (2 ml.) with stirring. A precipitate was formed immediately and after 15 minutes the pH was adjusted to 7–8 by the addition of ammonium hydroxide and the product collected. The *azapurine* formed colourless

needles, m.p. 188 to 189°, from aqueous methanol. (Found: C, 58·3; H, 4·6; N, 36·8. $C_{11}H_{10}N_6$ requires C, 58·4; H, 4·5; N, 37·1 per cent).

C. 2-Amino-9(4′-chlorophenyl)8-azapurine

(i) *2-Chloro-4-(4′-chloroanilino)-5-nitropyrimidine.* 2:4-Dichloro-5-nitropyrimidine (1·0 g.) in ethanol (25 ml.) was cooled to −10° in an ice-salt bath and treated all at once with a solution of *p*-chloroaniline (0·66 g.) in ethanol (12 ml.). After a few seconds the clear solution became a mass of yellow solid, which was immediately collected and recrystallised from ethanol, to yield *2-chloro-4(4′-chloroanilino)-5-nitropyrimidine* as yellow needles, m.p. 150 to 151°. (Found: C, 42·0; H, 2·6; N, 19·65; Cl, 24·5. $C_{10}H_6O_2N_4Cl_2$ requires C, 42·1; H, 2·1; N, 19·7; Cl, 24·9 per cent).

If the reaction was attempted on a larger scale, then a mixture was obtained with the di-*p*-chloroanilino compound. For comparison an authentic sample of this was prepared from the dichloronitropyrimidine (1 g.) in ethanol (15 ml.) and *p*-chloroaniline (2·64 g.) in ethanol (50 ml.), the pyrimidine being added to the amine this time. The mixture was then boiled under reflux to complete the reaction and the product, isolated by filtration, formed pale yellow needles, m.p. 216 to 217° from *n*-butanol. (Found: C, 51·3; H, 3·0; N, 18·8; Cl, 19·3. $C_{16}H_{11}O_2N_5Cl_2$ requires C, 51·0; H, 2·9; N, 18·6; Cl, 18·9 per cent).

2-Amino-4(4′-chloroanilino)-5-nitropyrimidine. 2-Chloro-4(4′-chloroanilino)-5-nitropyrimidine (2·0 g.) was heated at 100° for three hours in a sealed tube with saturated ethanolic ammonia solution (20 ml.). The product (1·9 g.), collected on cooling, crystallised from *n*-butanol as golden-yellow leaflets, m.p. 241°. (Found: C, 45·5; H, 3·45; N, 26·3; Cl, 13·6. $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2:5-Diamino-4(4′-chloroanilino)pyrimidine. The above nitro compound (1·32 g.) was added to a solution of stannous chloride (5·0 g.) in concentrated hydrochloric acid (25 ml.) and the mixture warmed and ground. When reduction was complete, as shown by the replacement of the orange colour by a cream suspension of the stannichloride, the product was collected, decomposed by 40 per cent sodium hydroxide and the base collected by extraction with chloroform. The residue obtained by evaporation of the extract was crystallised from hot 2N hydrochloric acid (charcoal) to yield the *dihydrochloride monohydrate* as colourless needles, m.p. 286 to 287° (decomp.) (Found: C, 37·1; H, 4·5; N, 21·8; Cl, 32·7. $C_{10}H_{10}N_5Cl{\cdot}2HCl{\cdot}H_2O$ requires C, 36·75; H, 4·3; N, 21·45; Cl, 32·65 per cent). The *monopicrate* formed lemon-yellow rosettes of prisms, m.p. 256° (decomp.) from aqueous ethanol. (Found: C, 41·8; H, 3·1; N, 23·9; Cl, 7·9. $C_{10}H_{10}N_5Cl{\cdot}C_6H_3O_7N_3$ requires C, 41·3; H, 2·8; N, 24·1; Cl, 7·65 per cent).

2-Amino-9(4′-chlorophenyl)-8-azapurine (CB. 2356, No. 63)

2:5-Diamino-4(4′-chloroanilino)pyrimidine dihydrochloride (0·4 g.) was dissolved in water (80 ml.), concentrated hydrochloric acid (2 drops)

was added, and the mixture cooled to 5° in ice-water. A solution of sodium nitrite (0·12 g.) in water (1 ml.) was added and the mixture was well stirred. After adjustment of the pH to 8 by means of concentrated ammonium hydroxide, the precipitate was collected and crystallised from aqueous methanol to afford the triazolopyrimidine as rosettes of fine white needles, m.p. 236°. (Found: C, 48·7; H, 3·0; N, 34·2; Cl, 14·7. $C_{10}H_7N_6Cl$ requires C, 48·7; H, 2·8; N, 34·1; Cl, 14·4 per cent).

8-*Azapurine* (CB. 2354, No. 64)

4:5-Diaminopyrimidine (0·4 g.) in 95 per cent ethanol (10 ml.) was refluxed with amyl nitrite (5·5 ml., 10 mol.) for two hours, by which time the solution was nearly clear. It was cooled, filtered from a small amount of dark coloured matter which was discarded and the filtrate was evaporated to dryness to leave a brown solid. This was sublimed at 130 to 140°/1·5 mm. to yield a cream substance, smelling slightly of fatty acids. The m.p. varied with the rate of heating and standard conditions had to be adopted to obtain concordant results. The sample was placed in an electrically-heated copper block at 125° and the temperature raised at the rate of 2° per minute. The m.p. was then 170 to 171°.

The product was resublimed at 130 to 140°/1·5 mm. and the initial fraction (0·08 g.) m.p. 164 to 165° was rejected. Sublimation was continued and the second fraction (0·29 g.) had m.p. 175°. A small amount of yellowish substance was obtained subsequently and the remainder consisted of brown, involatile decomposition products. Repeated sublimation of the fraction, m.p. 175° only gave material of the same m.p. and an involatile residue. The final *sublimate* was a white solid, insoluble in ether, but very soluble in water to give a solution with a strongly acid reaction to litmus. On treatment with silver nitrate solution, this gave a heavy white precipitate of the silver salt; with sodium bicarbonate solution a vigorous effervescence is observed. (Found: C, 39·9; H, 2·6; N, 58·1; $C_4H_3N_5$ requires C, 39·7; H, 2·5; N, 57·8 per cent).

2:6-*Diamino*-3(4′-*chlorophenylazo*)*pyridine* (CB. 2357, No. 66)

2:6-Diaminopyridine (5·45 g.) in dilute hydrochloric acid solution (2N, 50 ml.) was cooled in ice, stirred and treated slowly with a solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (6·375 g.)). To the resulting dark-red solution was added crystalline sodium acetate to adjust the pH to 5–6 and the thick yellow slurry so obtained was diluted with water and filtered. The product on crystallisation from aqueous ethanol afforded the *azo compound* as yellow prisms, m.p. 184 to 185°. (Found: C, 53·45; H, 4·3; N, 27·9; Cl, 14·1. $C_{11}H_{10}N_5Cl$ requires C, 53·4; H, 4·0; N, 28·3; Cl, 14·3 per cent).

Biological Section*

Methods

Strains. Streptococcus faecalis (R) was used for experiments with pteroylglutamic acid (PGA) and *Leuconostoc citrovorum* (NCTC 7837)

* By H.O.J.C. and P.L.H.

with 5-formyltetrahydropteroylglutamic acid (folinic acid). Stock cultures of *Str. faecalis* were maintained in the liver-tryptone agar of Nymon and Gortner[20], and stock cultures of *Leuc. citrovorum* in brewer's yeast agar. Tests were carried out in a basal medium similar to that used by Collier and Phillips[6] for testing antagonists of folinic acid, which was based on the medium of Barton-Wright, Emery and Robinson[21]. This contained: acid hydrolysed casein (A. & H.), 6 g.; L-cystine, 100 mg.; DL-tryptophane, 120 mg.; glucose, 10 g.; sodium acetate (anhydrous), 10 g.; adenine HCl, 10 mg.; guanine HCl, 10 mg.; uracil, 10 mg.; xanthine, 10 mg.; aneurine, 100 μg.; riboflavine, 200 μg.; pyridoxine HCl, 100 μg.; calcium D-pantothenate, 500 μg.; D-biotin, 0·4 μg.; nicotinic acid, 100 μg.; NaCl, 5 g.; KH_2PO_4, 500 mg.; K_2HPO_4, 500 mg.; $MgSO_4 \cdot 7H_2O$, 200 mg.; $MnSO_4 \cdot 4H_2O$, 10 mg.; $FeCl_3$, 2 mg.; glass-distilled water, to 500 ml. The pH of this medium was adjusted to 7·0 and after steaming for 30 minutes the medium was filtered while hot.

Antifolic tests. The above medium, which was double-strength, was filled out in 5 ml. aliquots, in which solutions of test compounds were serially diluted. PGA was dissolved in a little potassium dihydrogen phosphate solution and made up to the required concentration in glass-distilled water. Five ml. quantities of PGA solution were added to aliquots of medium before autoclaving at 10 lb. for 10 min. Folinic acid was dissolved in sterile glass-distilled water and added with sterile precautions to double-strength medium after autoclaving. Inocula of bacteria were prepared from stock cultures by growing in liver tryptone broth for 20 hours at 37°. Cultures were centrifuged, washed twice with sterile saline and made up to correspond in opacity with Brown's No. 2 tube. This suspension was diluted 1 : 100 with saline and 0·02 ml. used as inoculum. After 60 to 64 hour incubation at 37°, growth was estimated by titration with 0·1N NaOH, using bromthymol blue as indicator. For reference, 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 2 : 4-diamino-6 : 7-di*iso*propylpteridine (0/129) were used.

EXPERIMENTAL

Str. faecalis *and pteroylglutamic acid.* The technique of screening tests is illustrated in Figure 1. When *Str. faecalis* was cultivated in the presence of enough PGA to give good growth (2 ng./ml.), increasing concentrations of an inhibitor depressed growth to an increasing degree. When the level of PGA was raised to 20 ng./ml., higher concentrations of inhibitor were required to produce equal depression, indicating some degree of competitive antagonism.

The antifolic acid activities of all compounds described in the chemical section were examined by this method, using levels of 2 and 20 ng./ml. PGA. Compounds numbered 9, 12, 15, 19, 21, 22, 25, 29, 32–34, 49–58, 60–66, and 68 were inactive in saturated solution, while the remainder showed activity. The concentration of each active inhibitor required to depress growth by half at each level of PGA was determined in three or more independent experiments. The geometric means of these concentrations and their 95 per cent fiducial limits, together with the molar ratios

of inhibitor to PGA based on these mean values, and the ratios of the mean inhibitory concentrations at 20 and 2 ng./ml. PGA are expressed for aryl-azopyrimidines in Table III. Corresponding results for 8-azapurines, Compound 67, A-methopterin and pteridine 0/129 are given in Table IV. From these Tables it will be seen that the most active compounds among azopyrimidines were Nos. 5, 6, 11, 17 and 30 and among azapurines the considerably more potent Nos. 45 and 48. Compound 45 had the highest potency, which was about one-fiftieth that of A-methopterin in the same test.

Leuc. citrovorum *and folinic acid.* Replacing *Str. faecalis* by *Leuc. citrovorum*, some of the compounds antagonising PGA were screened at levels of 0·92, 4·6 or 23 ng./ml. folinic acid. Of the aryl-azopyrimidines tested, only compounds 1, 13 and 26 showed some degree of competitive antagonism, the remainder tested (Nos. 2, 3, 4, 6, 8, 10, 14, 16, 17, 20, 23, 24 and 27) being inactive. All the azapurines tested (Nos. 35, 37, 38, 40, 42 and 48) were inactive and so was Compound 67. A-methopterin had about one-hundredth and Compound 1 about one-fifth of their respective potencies with *Str. faecalis.*

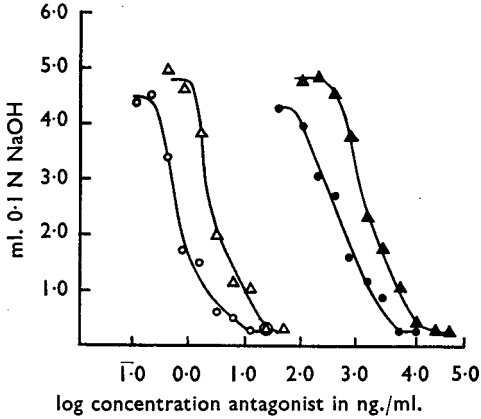

FIG. 1. Growth inhibition of *Str. faecalis* in the presence of pteroylglutamic acid (PGA).
● Compound 1 at 2 ng./ml. PGA
▲ ,, ,, ,, 20 ,, ,,
○ A-methopterin at 2 ng./ml. PGA
△ ,, ,, 20 ,, ,,

DISCUSSION

In 1914 Langley[22] showed that for any particular muscle of the frog, the ratio of curare (antagonist) to nicotine (agonist) necessary to prevent a response was constant and independent of the levels of concentration. In 1940, a similar constancy of ratio between sulphanilamide and aminobenzoic acid for *Str. pyogenes* was described by Woods[23], who ascribed it to competition between those substances for an enzyme. Since then, constancy of ratio between antagonist and agonist has commonly been used as a test of competition, particularly in bacteria. Schild[24], however has argued that "the mass action equation as developed by Gaddum[25] for a first order reaction requires a ninefold increase of antagonist corresponding to a five-fold increase of active drug. . . . Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation". The above divergent viewpoints and the work of Timms[26] suggest that methods based simply on measuring ratios of antagonist to

growth-factor required for a given biological effect do not fully characterise competitive action. Such methods however can indicate some degree of competition.

It will be seen from Tables III and IV that for each compound a tenfold increase in PGA requires a twofold to eightfold increase in inhibitor to produce the same degree of inhibition. This indicates the presence of

TABLE III

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS ARYL-AZOPYRIMIDINES IN THE PRESENCE OF PTEROYLGLUTAMIC ACID (PGA). VALUES EXPRESSED AS GEOMETRIC MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		μg./ml. to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng./ml. PGA
		2 ng./ml. PGA		20 ng./ml. PGA				
No.	CB Reference No.	Mean	Limits	Mean	Limits	2 ng./ml.	20 ng./ml.	
1	2295	0·33	0·20–0·56	1·66	1·34–2·07	317·8	159·8	5·0
2	2310	0·25	0·09–0·67	1·13	0·39–3·26	209·2	94·55	4·5
3	2309	0·39	0·15–1·06	1·06	0·41–2·74	326·3	88·70	2·7
4	2277	0·21	0·08–0·59	1·08	0·45–2·61	175·7	90·37	5·1
5	2328	0·19	0·12–0·28	0·82	0·35–1·92	136·0	58·70	4·3
6	2311	0·17	0·12–0·23	0·81	0·60–1·09	121·7	57·98	4·8
7	2326	0·57	0·16–2·08	2·75	0·99–7·67	424·2	203·5	4·8
8	2307	0·41	0·14–1·17	0·71	0·50–1·00	303·4	52·53	1·7
10	2283	0·54	0·20–1·43	2·22	0·80–6·18	399·6	164·3	4·1
11	2349	0·27	0·11–0·65	0·54	0·25–1·19	153·8	30·76	2·0
13	2299	1·11	0·51–2·41	5·00	1·96–12·76	945·0	425·7	4·5
14	2323	3·67	1·30–10·35	12·88	4·60–36·08	2,964	1,040	3·5
16	2330	0·28	0·12–0·67	0·86	0·31–2·39	221·3	67·96	3·1
17	2315	0·16	0·06–0·45	0·43	0·21–0·90	129·4	33·98	2·7
18	2331	2·26	1·01–5·06	4·77	2·10–10·77	1,779	375·6	2·1
20	2298	12·28	7·25–20·81	—*	—	9,568	—	—
23	2271	22·41	10·95–45·84	7·60	28·45–49·68	13,650	2,290	1·7
24	2266	7·72	3·08–19·36	—*	—	5,840	—	—
26	2280	14·59	7·02–30·34	22·40	12·66–39·64	9,153	1,405	1·6
27	2288	0·49	0·38–0·64	1·10	0·46–2·63	408·5	91·70	2·3
28	2336	0·76	0·53–1·09	1·65	1·15–2·36	542·3	117·7	2·2
30	2329	0·13	0·05–0·39	0·54	0·27–1·09	115·3	47·90	4·2
31	2304	20·72	18·02–24·39	52·94	27·50–102·0	18,310	4,679	2·6

* Incomplete or no inhibition in saturated solution.

TABLE IV

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS 8-AZAPURINES AND OTHER COMPOUNDS IN THE PRESENCE OF PTEROYLGLUTAMIC ACID (PGA). VALUES EXPRESSED AS GEOMETRIC MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		μg./ml. to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng./ml. PGA
		2 ng./ml. PGA		20 ng./ml. PGA				
No.	CB Reference No.	Mean	Limits	Mean	Limits	2 ng./ml.	20 ng./ml.	
35	2314	1·16	0·62–2·15	5·43	4·24–6·93	1,126	527·5	4·7
36	2321	0·35	0·13–0·93	1·96	1·66–2·32	295·1	165·3	5·6
37	2308	0·62	0·26–1·52	4·68	1·75–12·51	522·9	394·6	7·6
38	2278	0·12	0·07–0·21	0·85	0·36–2·03	101·2	71·67	7·1
39	2332	0·46	0·26–0·84	1·83	1·53–2·18	331·5	131·9	4·0
40	2319	0·12	0·05–0·30	0·59	0·28–1·24	86·47	42·52	4·9
41	2333	0·22	0·09–0·58	0·73	0·34–1·58	163·9	54·38	3·3
42	2318	0·12	0·05–0·29	0·57	0·24–1·37	83·39	42·46	4·8
43	2324	0·19	0·12–0·28	1·20	0·44–3·28	141·5	89·39	6·3
44	2282	0·22	0·08–0·63	1·42	0·53–3·78	163·9	105·8	6·5
45	2364	0·04	0·02–0·10	0·11	0·04–0·30	22·90	6·30	2·8
46	2350	0·51	0·41–0·64	1·64	0·58–4·65	242·4	77·93	3·2
47	2327	0·98	0·37–2·63	2·71	0·98–7·45	797·4	220·5	2·8
48	2335	0·04	0·03–0·07	0·19	0·09–0·38	31·84	15·12	4·8
59	2381	0·10	0·04–0·24	0·42	0·22–0·81	79·32	33·32	4·2
67	2316	0·57	0·24–1·36	3·89	2·67–5·66	897·8	612·7	6·7
A-Methopterin		0·0005	0·0002–0·0012	0·0023	0·0013–0·0041	0·226	0·112	4·6
Pteridine 0/129		0·0026	0·0011–0·0060	0·0143	0·0071–0·0290	2·301	1·286	5·5

some degree of competitive antagonism of pteroylglutamic acid. A full test of competition depends on analysis of curves of log concentration of folic acid plotted against bacterial growth in the absence of and in the presence of various levels of inhibitor. Such curves have shown that Compound 1 competes with both PGA and folinic acid. These curves and others at present being obtained for further compounds will be published elsewhere.

Structure-Activity Relations

The fact that the 4-amino-5-aryl-azopyrimidines (III) can be readily oxidised, by chemical agents, to the 8-aryl-8-azapurines (IV) and that both series of compounds contain active substances suggested that the activity of the azo compounds might be due to this conversion by oxidation during bacterial metabolism. Detailed investigation however revealed a marked lack of the relation which, on this hypothesis, would have been expected between the activities of corresponding compounds in the two series. For example, for *Str. faecalis* and PGA, certain 8-azapurines (Nos. 49, 50 and 55) which are substituted with amino and hydroxy groups at the 2 and 4 positions (IV, $R'' = NH_2$, $R' = OH$) respectively, are inactive, whilst the corresponding azo compounds (Nos. 27, 28 and 31) are active. Again for *Leuc. citrovorum* and folinic acid the 8-azapurine No. 35 is inactive, but the corresponding azopyrimidine (No. 1) is active. On the other hand, for *Str. faecalis*, certain highly active 8-azapurines, e.g., Nos. 43 and 46, correspond to the inactive azopyrimidines, Nos. 9 and 12 respectively. A further discrepancy is revealed by comparison of the activities of three azopyrimidines (Nos. 2, 3 and 4), which differ only in the position of the chlorine atom in the benzene ring, and the corresponding 8-azapurines (Nos. 36, 37 and 38). The azo compounds are of comparable activity whilst the 8-azapurines showed more fluctuation.

An alternative hypothesis for the activity of the azopyrimidines was that they might be reductively split (a process known to occur *in vivo* with a variety of azo compounds[27–30]) to yield a 4:5-diaminopyrimidine (V) which might be the active form; but, although 2:4:5:6-tetraminopyrimidine (V, $R = NH_2$, No. 67), which might be derivable from many of the active compounds, was an antifolic acid, the inactivity of 2:4:5-triamino-6-hydroxypyrimidine (V, $R = OH$, No. 68), also derivable from the active azopyrimidines Nos. 27 and 31, rendered the hypothesis untenable. We conclude therefore that the antifolic activity found in the azopyrimidines and 8-azapurines is intrinsic for each series.

Simplification of the diamino-8-aryl-8-azapurines by removing the aryl substituent to produce 2:6-diamino-8-azapurine (No. 65) or by further removing the amino groups to give 8-azapurine itself (VI, No. 64) led to inactive compounds. The inactivity of No. 65 thus suggests that an aryl substituent may be necessary. Since, also, the three 9-aryl-8-azapurines prepared (VII, $R' = H$, $R'' = C_6H_5$, No. 61; $R' = CH_3$, $R'' = C_6H_5$, No. 62; $R' = H$, $R'' = C_6H_4Cl\,p$, No. 63) were all inactive, it may be that the particular position of the aryl substituent in the 8-aryl-8-azapurine structure relative to the pyrimidine ring is also important. As a flat

structure this substituent might be concerned in facilitating attachment to an enzyme. Modification of the two active structures by substitution of a pyrimidine by a pyridine ring as in VIII (No. 60) and IX (No. 66) led to inactivity.

In the aryl-azopyrimidines (III) activity is consistent with either one, two or three amino substituents in the pyrimidine ring, but for maximal effect three amino groups are generally desirable. The effects of substitution in the benzene ring with chlorine or bromine depend on the position and number of the substituent atoms. A single atom in the *para* position was beneficial, but dihalogen compounds were usually less active than the mono-substituted and the tribromo compound (No. 12) was inactive.

Of the twenty-five 8-aryl-8-azapurines tested, fifteen contained two amino groups in the pyrimidine ring and all were active. The remaining ten compounds contained one or no amino groups. Since none of these was active, we conclude that two amino groups are essential for activity in this series. In the azapurines, the effects of substitution in the benzene ring with chlorine or bromine differed in some ways from those in the azopyrimidine series. Substitution of a single halogen atom in any position increased activity, and the dihalogen compounds were generally more active than the monosubstituted. On the other hand, as in the azopyrimidine series, the *para* position in the monosubstituted derivatives was the most beneficial, and the tribromo derivative (No. 46) was much less active than the dibromo compound (No. 45).

The active compounds of the 8-azapurine series, some of which are more potent than any of the azopyrimidines tested, provide what appear to be the first examples of antifolic acid activity among 8-azapurines.

Summary

1. Thirty-four amino-5-aryl-azopyrimidines and thirty amino-8-aryl-8-azapurines and a few examples of three related structures were synthesised as potential antifolic acids because of a structural relation to pteridine.

2. Screened with *Str. faecalis* and pteroylglutamic acid (PGA), 23 aryl-azopyrimidines and fifteen 8-azapurines showed antifolic acid activity. This was greatest in Compound 45, which possessed about one-fiftieth the potency of A-methopterin in the same test.

3. With *Leuc. citrovorum* and folinic acid, 3 of 16 azopyrimidines and none of 6 azapurines tested showed antifolic acid activity. A-methopterin had about one-hundredth and Compound 1 about one-fifth of their respective potencies with *Str. faecalis*.

Consideration of the activities of the above compounds has enabled certain structure-activity relations to be deduced.

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REFERENCES

1. Burchenal, Robinson, Johnston and Kushida, *Science*, 1950, **111**, 116.
2. Edeson and Field, *Brit. med. J.*, 1950, **1**, 88.
3. Daniel, Norris, Scott and Heuser, *J. biol. Chem.*, 1947, **169**, 689.
4. Daniel and Norris, *ibid.*, 1947, **170**, 747.
5. Collier, Campbell and Fitzgerald, *Nature, Lond.*, 1950, **165**, 1004.
6. Collier and Phillips, *ibid.*, 1954, **174**, 180.
7. Felton and Timmis, Resumés des Communications, XIVth Congrès International de Chimie pure et appliquée, Zurich, 1955, p. 228.
8. Modest, Schlein and Foley, *J. Pharm. Pharmacol.*, 1957, **9**, 68.
9. Spickett and Timmis, *J. chem. Soc.*, 1954, 2887.
10. Cf. Hartley, *ibid.*, 1938, 633.
11. Foley, *Proc. Soc. exp. Biol. N.Y.*, 1953, **83**, 733, 740, 742.
12. Benson, Hartzel and Savell, *ibid.*, 1950, **72**, 1816.
13. Falco, Hitchings and Russell, *J. Amer. chem. Soc.*, 1949, **71**, 362.
14. Cavalieri, Tinker, Bendich and Brown, *ibid.*, 1948, **70**, 3875.
15. Traube, *Ber.*, 1900, **33**, 1377.
16. Traube, *ibid.*, 1904, **37**, 4546.
17. Brown, *J. Soc. chem. Ind.*, 1950, **69**, 353.
18. Lythgoe, Todd and Topham, *J. chem. Soc.*, 1944, 315.
19. Spickett, Ph.D. Thesis, London, 1952.
20. Nymon and Gortner, *J. biol. Chem.*, 1946, **163**, 277.
21. Barton-Wright, Emery and Robinson, *Biochem. J.*, 1945, **39**, 334.
22. Langley, *J. Physiol.*, 1914, **48**, 73.
23. Woods, *Brit. J. exp. Path.*, 1940, **21**, 74.
24. Schild, *Brit. J. Pharmacol.*, 1947, **2**, 189.
25. Gaddum, *J. Physiol.*, 1937, **89**, 7P.
26. Timms, *Brit. J. Pharmacol.*, 1956, **11**, 273.
27. Elson and Warren, *Biochem. J.*, 1944, **38**, 217.
28. Fuller, *Lancet*, 1937, **1**, 194.
29. Sisley and Porcher, *C.R. Acad. Sci., Paris*, 1911, **152**, 1062.
30. Stevenson, Dobriner and Rhoads, *Cancer Res.*, 1942, **2**, 160.